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Exploring the venom of the forest cobra snake: Toxicovenomics and antivenom profiling of *Naja melanoleuca*

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Abstract

A toxicovenomic analysis of the venom of the forest cobra, *N. melanoleuca*, was performed, revealing the presence of a total of 52 proteins by proteomics analysis. The most abundant proteins belong to the three-finger toxins (3FTx) (57.1 w%), which includes post-synaptically acting α -neurotoxins. Phospholipases A₂ (PLA₂) were the second most abundant group of proteins (12.9 w%), followed by metalloproteinases (SVMPs) (9.7 w%), cysteine-rich secretory proteins (CRISPs) (7.6 w%), and Kunitz-type serine proteinase inhibitors (3.8 w%). A number of additional protein families comprised each less than 3 w% of venom proteins. A toxicity screening of the fractions, using the mouse lethality test, identified toxicity in RP-HPLC peaks 3, 4, 5 and 8, all of them containing α -neurotoxins of the 3FTx family, whereas the rest of the fractions did not show toxicity at a dose of 0.53 mg/kg. Three polyspecific antivenoms manufactured in South Africa and India were tested for their immunoreactivity against crude venom and fractions of *N. melanoleuca*. Overall, antivenoms immunorecognized all fractions in the venom, the South African antivenom showing a higher titer against the neurotoxin-containing fractions. This toxicovenomic study identified the 3FTx group of α -neurotoxins in the venom of *N. melanoleuca* as the relevant targets to be neutralized.

(200 words)

Biological significance

A toxicovenomic analysis of the venom of the forest cobra, also known as black cobra, *Naja melanoleuca*, was performed. Envenomings by this elapid species are characterized by a progressive descending paralysis which starts with palpebral ptosis and, in severe cases, ends up with respiratory arrest and death. A total of 52 different proteins were identified in this venom. The most abundant protein family was the three-finger toxin (3FTx) family, which comprises almost 57.1 w% of the venom, followed by phospholipases A₂ (PLA₂) (12.9 w%). In addition, several other protein families were identified in a much lower percentage in the venom. A toxicity screening of the fractions, using the mouse lethality assay, identified four peaks as those having toxicity higher than that of the crude venom. These fractions predominantly contain α -neurotoxins of the 3FTx family. This toxicovenomic characterization agrees with the clinical and experimental manifestations of envenomings by this species, in which a strong neurotoxic effect predominates. Therefore, our findings suggest that immunotherapy against envenomings by *N. melanoleuca* should be directed towards the neutralization of 3FTxs; this has implications for the improvement of current antivenoms and for the development of novel antivenoms based on biotechnological approaches. A screening of the immunoreactivity of three antivenoms being distributed in sub-Saharan Africa revealed that they immunoreact with the fractions containing α -neurotoxins, although with different antibody titers.

1. Introduction

The forest cobra, also known as the black cobra (*Naja melanoleuca*), is a highly venomous member of the elapid snake family, reaching up to 3.1 meters in length, and being able to deliver venom yields above 1 gram per milking [1]. *N. melanoleuca* is the largest of the African cobra species and it is known to inhabit moist river areas, primary and secondary forests, and suburban habitats in Western, Central, and Southern Africa [2–4]. Its coloration may vary between three different color morphs, and it is active during the day, where it feeds on mammals, frogs, and fish [2,3]. From the clinical standpoint, envenomings by *N. melanoleuca* have been classified within the syndromic category 3, characterized by progressive paralysis (neurotoxicity) [5]. Patients develop a descending progressive paralysis which starts with ptosis, external ophtalmoplegia and weakness of muscles innervated by the cranial nerves, with patients having difficulties in swallowing and speaking. Eventually the respiratory muscles become paralyzed, and death ensues unless mechanical ventilation is provided [5].

Currently, six antivenoms are claimed to be effective against envenomings from *N. melanoleuca* [6]. Due to the severity of envenomings, *N. melanoleuca* is classified by the WHO as a category 1 snake of highest medical importance (<http://apps.who.int/bloodproducts/snakeantivenoms/database/>). Therefore, it is of high relevance to obtain a deep understanding of the composition of *N. melanoleuca* venom. To this date, no quantitative venom proteome has been reported for *N. melanoleuca*, however, several biochemical studies have reported that the venom contains long and short neurotoxins [7,8], cytotoxins [9–11], phospholipases A₂ [12,13], and 'weak' toxins [9,14].

In order to develop safe and effective antivenoms that can protect against envenoming from *N. melanoleuca*, it is not only important to know the venom composition. It is also essential to understand which toxins are the medically most relevant to target. For this purpose, the combination of venomomics and the Toxicity Score [15] may be employed to unveil which toxins are the main culprits responsible for the clinical manifestations of *N. melanoleuca* envenomings. Being able to identify these key toxins may not only help guide traditional antivenom development, but may also aid rational antitoxin discovery approaches based on biotechnology [16].

Here, we report the first toxicovenomics study of the venom of *N. melanoleuca*, providing a quantitative estimation of its proteome alongside an assessment of the medical importance of the individual venom fractions and an evaluation of the immunorecognition pattern of three antivenoms in use in sub-Saharan Africa.

2. Materials and Methods

2.1 Snake venom

Venom of *N. melanoleuca* was obtained from Latoxan SAS, Valence, France, from a pool of 7 specimens collected in Uganda. Venoms from *N. nigricollis* and *N. mossambica* used for comparison in *in vitro* enzymatic assays were also obtained from Latoxan from pools of several specimens collected in Tanzania. Venom from *Bothrops asper* was obtained as a pool from several specimens from Costa Rica kept at Instituto Clodomiro Picado, Universidad de Costa Rica, Costa Rica.

2.2 Venom separation by reverse-phase HPLC and SDS-PAGE

Following the ‘snake venomomics’ analytical strategy, crude venom was

fractionated involving a combination of RP-HPLC and SDS-PAGE separation [17]. Two mg of venom was dissolved in 200 μ L of water containing 0.1% trifluoroacetic acid (TFA; solution A) and separated by RP-HPLC (Agilent 1200) on a C₁₈ column (250 x 4.6 mm, 5 μ m particle; Supelco). Elution was carried out at 1 mL/min by applying a gradient towards solution B (acetonitrile, containing 0.1% TFA): 0% B for 5 min, 0–15% B over 10 min, 15–45% B over 60 min, 45–70% B over 10 min, and 70% B over 9 min, as previously described [18]. Fractions were collected manually, dried in a vacuum centrifuge, redissolved in water, reduced with 5% β -mercaptoethanol at 100°C for 5 min, and further separated by SDS-PAGE in 15% gels. Colloidal Coomassie blue G-250 was used for proteins staining, and a ChemiDoc[®] recorder and ImageLab[®] software (Bio-Rad) were used to acquire gel images.

2.3 Protein identification by tandem mass spectrometry of tryptic peptides

From the polyacrylamide gels protein bands were excised and subjected to reduction (10 mM dithiothreitol), alkylation (50 mM iodoacetamide), and overnight in-gel digestion with sequencing grade trypsin (Sigma), in 50 mM ammonium bicarbonate at 37 °C. Tryptic peptides were extracted with 50% acetonitrile containing 1% TFA, and analyzed by MALDI-TOF-TOF on an AB4800-Plus Proteomics Analyzer (Applied Biosystems). Digested and extracted peptides were mixed with an equal volume of saturated α -cyano-hydroxycinnamic acid (in 50% acetonitrile, 0.1% TFA), and spotted (1 μ L) onto an Opti-TOF 384-well plate, dried, and analyzed in positive reflector mode. TOF spectra were acquired using 500 shots at a laser intensity of 3000. TOF/TOF fragmentation spectra were acquired using 500 shots at a laser intensity of 3900 for the automatically selected ten most intense

precursor ions. CalMix[®] standards (ABSciex) spotted onto the same plate were used for external calibration in each run. Resulting spectra were searched against the UniProt/SwissProt database for Serpentes (20150217) using ProteinPilot[®] v.4 and the Paragon[®] algorithm (ABSciex) at $\geq 95\%$ confidence, or, in few cases, manually interpreted, and the deduced sequences searched using BLAST (<http://blast.ncbi.nlm.nih.gov>) for assignment of protein family by similarity.

2.4 Relative protein abundance estimations

The relative abundance of the venom proteins was estimated using the ChemStation[®] software (Agilent) to integrate the areas of their chromatographic peaks at a wavelength of 215 nm, roughly corresponding to peptide bond abundance [17]. When HPLC peaks contained several electrophoretic bands, ImageLab[®] (Bio-Rad) was used to assign their percentage distributions by densitometry. Finally, for electrophoretic bands containing more than one protein according to MALDI-TOF-TOF analysis, their percentage distributions were estimated based on the corresponding intensities of the intact protein ions, as observed in the nESI-MS analysis. For this, a 10 μ L sample of the HPLC fraction was loaded into a metal-coated capillary (Proxeon) and directly infused into a nano-spray source of a QTrap 3200 mass spectrometer (Applied Biosystems) operated at 1300 V in enhanced multi-charge mode. Deconvolution of spectra was performed with the aid of the Bayesian protein reconstruction tool of Analyst v.1.5. Intensities lower than 5% (relative to the major protein ions in these mixtures) were considered as traces. Protein abundances were calculated on the basis of protein content percentage (w%).

2.5 In vitro enzymatic activities

2.5.1. Phospholipase A₂ activity

PLA₂ activity was assayed using the monodisperse synthetic chromogenic substrate 4-nitro-3-octanoyloxybenzoic acid (NOBA) [19]. 25 μ L of solution, containing various amounts of venom, were mixed with 200 μ L of 10 mM Tris, 10 mM CaCl₂, 0.1 M NaCl, pH 8.0, and 25 μ L of NOBA to achieve a substrate concentration of 0.32 mM. Plates were incubated at 37 °C for 60 min, and absorbances were recorded at 405 nm (Multiskan FC, Thermo Scientific). For comparative purposes, the activities of the venoms of *N. mossambica*, *N. nigricollis*, and the viperid snake *Bothrops asper* were also assessed.

2.5.2 Proteinase activity

Proteinase activity was assayed by adding 20 μ g of venom to 100 μ L of azocasein (10 mg/mL in 50 mM Tris–HCl, 0.15 M NaCl, 5 mM CaCl₂ buffer, pH 8.0), and the mixture was incubated for 90 min at 37 °C. The reaction was terminated by addition of 200 μ L of 5% trichloroacetic acid. After centrifugation (5 min, 6,000 \times g), 150 μ L of supernatants were mixed with 100 μ L of 0.5 M NaOH, and absorbances were recorded at 450 nm (Multiskan FC, Thermo Scientific). The absorbance of azocasein incubated with distilled water alone was used as a blank, being subtracted from all readings [20]. For comparative purposes, the activities of the venoms of *N. mossambica*, *N. nigricollis*, and *Bothrops asper* were also assessed.

2.6 Toxicological profiling

2.6.1 Animals

In vivo assays were performed in CD-1 mice of both sexes, provided by Instituto Clodomiro Picado, following protocols approved by the Institutional

Committee for the Use and Care of Animals (CICUA), University of Costa Rica.

Mice were provided food and water *ad libitum*.

2.6.2 Toxicity of crude venom and isolated venom fractions

The acute toxicity of venom fractions was initially screened by intravenous (i.v.) injection of 10 µg of toxin per mouse (0.53 mg/kg) for all fractions devoid of snake venom metalloproteinases (SVMPs) in groups of three mice (18–20 g body weight). Fractions that were not lethal at this level were not further investigated, whereas precise LD₅₀s were determined in groups of four mice for fractions which did kill mice at this dose, and for the whole venom. Toxicity Scores were calculated according to Laustsen et al. [15], on the basis of the abundance (w%) of each fraction. Additionally, a Molecular Toxicity Score was introduced, which was calculated using the molecular abundance (mol%) of each fraction based on the molecular mass of identified toxins. Various amounts of venom or venom fractions were dissolved in phosphate-buffered saline (PBS; 0.12 M NaCl, 0.04 M sodium phosphate buffer, pH 7.2) and injected in the caudal vein, in a volume of 100 µL. Deaths occurring within 24 h were recorded, and LD₅₀s were calculated by probits [21], using the BioStat[®] software (AnalySoft).

2.7 Antivenoms

Polyspecific antivenoms from the following manufacturers were employed: (a) SAIMR (South African Institute for Medical Research) Polyvalent Snake Antivenom from South African Vaccine Producers (Pty) Ltd (batch number BC02645, expiry date 07/2016); (b) Snake Venom Antivenom (Central Africa) from VINS Bioproducts

Ltd (batch 12AS13002, expiry date 04/2017); (c) Snake Venom Antivenom (African) from VINS Bioproducts Ltd (batch 13022, expiry date 01/2018).

2.8 Immunoreactivity of antivenoms against crude venom and venom fractions by ELISA

Wells in MaxiSorp™ plates (NUNC, Roskilde, Denmark) were coated with 1 µg of each HPLC venom fraction, or crude venom, dissolved in 100 µL PBS overnight. Next day, the wells were washed three times with PBS and blocked by adding 100 µL PBS containing 2% (w:v) bovine serum albumin (BSA, Sigma), and incubated at room temperature for 1 h. Then, plates were washed five times with PBS. A dilution of each antivenom in PBS + 2% BSA was prepared, and 100 µL of these solutions were added to each well in triplicates and incubated for 2 h. Plates were then washed five times with PBS. Subsequently, 100 µL of a 1:2000 dilution of conjugated antibody (Sigma A6063, rabbit anti-horse IgG (whole molecule)-alkaline phosphatase in PBS + 1% BSA) was then added to each well. Following 2 h of incubation, the wells were washed five times with FALC buffer (0.05 M Tris, 0.15 M NaCl, 20 µM ZnCl₂, 1 mM MgCl₂, pH 7.4). Color development was achieved by addition of 100 µL *p*-nitrophenyl phosphate (1 mg/mL in 9.7% v/v diethanolamine buffer, pH 9.8) and absorbances at 405 nm were recorded (Multiskan FC, Thermo Scientific).

3.0 Results and Discussion

3.1 Venomics

A bottom-up venomic characterization of *N. melanoleuca* venom was performed. Using RP-HPLC, the venom was resolved into 33 fractions, where the first, second and sixth fractions eluting from the column did not contain proteins as found in SDS-

PAGE. Further resolving by SDS-PAGE of the 30 remaining fractions yielded 63 bands (Figure 2), of which the protein identities for 62 bands were positively identified upon in-gel digestion and MALDI-TOF-TOF analysis. Within these bands a total of 52 different proteins were identified. As described previously [22,23], some fractions contained toxins in both monomer and dimer form, as exemplified by fraction number 10 (see Figure 2). To uncover the overall protein composition of *N. melanoleuca* venom, the identified proteins were assigned to families and expressed as percentages of total protein content (see Figure 3 and Table 1). The most abundant proteins were found to belong to the three-finger toxin (3FTx) family (57.1 w%), followed by proteins from the phospholipase A₂ (PLA₂) family (12.9 w%) (Figure 3). 3FTxs in elapid venoms share a common structural scaffold consisting of 60-80 amino acids, with three β -stranded loops extending from a small, globular, hydrophobic core [24–26]. Despite this highly conserved structure, 3FTxs display a wide range of activities [27], although the main 3FTxs identified in the venom of *N. melanoleuca* are type I and II α -neurotoxins and cytotoxins (see Table 1). Both type I and II α -neurotoxins target the nicotinic acetylcholine receptor at the end-plate of muscle fibers, causing flaccid paralysis in victims and prey, potentially culminating in respiratory failure and death [28–30]. The cytotoxins identified in this study derive from the type IA cytotoxin sub-subfamily. Cobra cytotoxins are amphiphilic toxins known for inducing cellular damage through disruption of the cell membrane, which may lead to tissue necrosis [31,32]. However, experimental and clinical envenomings by *N. melanoleuca* are not characterized by tissue necrosis possibly due to the relatively low content of cytotoxins as compared to the predominant α -neurotoxins.

The second-most abundant toxin family found in the venom of *N. melanoleuca* is the phospholipase A₂ (PLA₂) family (12.9 w%), which is generally found in

abundance in both viperids and elapids and, in the case of elapids, has evolved from pancreatic PLA₂ digestive enzymes [33]. The catalytic function of PLA₂s is to cleave phospholipids at the *sn*-2 position in the glycerol backbone. In snake venoms, however, PLA₂s not only play a role in the digestion of prey, but also exhibit a variety of toxicological effects [34]. As in other elapid venoms, the PLA₂s found in *N. melanoleuca* belong to the Group I, catalytically-active D49 enzymes [33]. In agreement, *N. melanoleuca* venom showed PLA₂ activity, similar to the venom of *N. nigricollis* but lower than that of venoms of *N. mossambica* and *Bothrops asper* (Figure 4A). Despite the presence of 9.7% SVMs in the venom proteome, very low proteinase activity was observed on azocasein (Figure 4B). It is likely that the SVMs of *N. melanoleuca* have restricted substrate specificity as observed in other elapid species [22,23]

Other protein families found in lower proportions in the venom of *N. melanoleuca* include cysteine-rich secretory proteins (CRISP; 7.6 w%), Kunitz-type serine proteinase inhibitors (BPTI/Kunitz; 3.8 w%), flavin monoamine oxidases (FMO; 2.5 w%), and traces of nerve growth factors (NGF < 0.4 w%), ohanin/vespryn family (OHA; < 0.5 w%), DNA/RNA endonucleases (DRE < 0.3 w%), endonuclease/phosphodiesterase (PDE; 1.1 w%), glutathione peroxidase family (GPF; 0.9 w%), type-B carboxylesterase/lipase family (CELF; 1.1 w%), and selectins (SUSHI; < 0.3 w%).

3.2. Toxicity of venom fractions

Toxicity testing was carried out for all venom fractions devoid of SVMs, since the solvents used for RP-HPLC denature these proteinases (Table 2). First, the LD₅₀ of crude venom was determined to be 0.66 mg/kg (95% confidence interval 0.49-0.92

mg/kg). This value was higher than previously determined LD₅₀s (i.v. 0.289 mg/kg), reported on <http://snakedatabase.org/pages/LD50.php>. It is likely that this can be explained by geographical variation in venom toxicity which is known to occur in species having a wide distribution [35]. A cut-off value of 10 µg per mouse (0.53 mg/kg) was chosen for toxicity screening of the venom fractions. From Table 2 it was evident that the majority of fractions inducing lethality within 24 h were those containing type I and II α-neurotoxins. LD₅₀s were determined for fractions that showed lethality at 0.53 mg/kg, more specifically fractions 3, 4, 5, and 8, as depicted in Table 2. These LD₅₀s were lower than the overall LD₅₀ of the crude venom suggesting that the toxins present in these fractions are of high medical relevance. According to their Toxicity Scores, the most potent fraction was fraction 8 (TS = 88, Molecular TS = 115.5), containing a homolog of Long neurotoxin OH-55 *Ophiophagus hannah* (Q53B58), Long neurotoxin 2 *Naja melanoleuca* (P01338), and Weak toxin S4C11 *Naja melanoleuca* (P01400). Previous studies have found Weak toxin S4C11 *Naja melanoleuca* (P01400) to have an LD₅₀ of 20 mg/kg [9], strongly suggesting that the long neurotoxins are the cause of the high toxicity of this fraction. From the MALDI-TOF-TOF analysis fractions 3, 4, and 5 were found to contain similar α-neurotoxins, with all three fractions having LD₅₀s in the same range. Due to differences in abundances, Toxicity Scores for these fractions differed (TS = 59.2, 4.6, and 10.0, Molecular TS = 78.8, 15.0, and 6.9, respectively). Nevertheless, the similarity in both the sequences obtained (Table 1) and the LD₅₀s (Table 2) suggest that the toxins present in these fractions are likely to be similar isoforms. No other toxins were found to show lethality at the pre-determined cut-off value. Mice injected with fractions 3, 4, 5, and 8 showed evident manifestations of respiratory paralysis, indicating that the cause of death was neurotoxin-induced respiratory arrest.

The toxicity analysis of fractions strongly suggests that other venom components, such as PLA₂s, do not play a central role in the overall toxicity. Despite the fact that neurotoxic PLA₂s occur in many elapid snake venoms [36], and although neurotoxic PLA₂s have been described in some *Naja* sp venoms [37], in general the neurotoxicity of cobra venoms is based on the action of post-synaptically acting 3FTxs. Likewise, other minor components present in *N. melanoleuca* venom do not seem to exert lethal effects either, as judged by our toxicity screening.

According to their Toxicity Scores, the most therapeutically relevant targets of *N. melanoleuca* to be neutralized with antivenom were found to be homologs of Alpha-neurotoxin NTX-1 from *N. sputratix* (Q9YGJ6), Short neurotoxin 1 from *N. melanoleuca* (P01424), Long neurotoxin 2 from *N. melanoleuca* (P01388), and Long neurotoxin OH-55 *O. hannah* (Q53B58) which are α -neurotoxins that bind to the nicotinic acetylcholine receptor, thereby abrogating neuromuscular transmission.

3.3 Immunoprofiling of antivenoms

Three polyspecific antivenoms, distributed in sub-Saharan Africa, were tested for their ability to recognize *N. melanoleuca* crude venom and fractions by ELISA. First, ELISA titration curves were determined against immobilized crude venom with the highest binding found to be VINS African, followed by SAVP, and VINS Central Africa, when normalized according to protein concentration (Figure 5). The immunization mixture of both VINS African and SAVP contain the venom of *N. melanoleuca*, whereas VINS Central Africa solely consists of venom from three species of the Viperidae family and *Dendroaspis polylepis*. Thus, cross-reactivity between similar toxins present in these species and *N. melanoleuca* is likely to exist.

When using solid-phase immunoassays of antivenoms against crude venoms, prediction of cross-reactivity may be of limited value as antibodies tend to bind to the highly immunogenic venom components, which are not always the most medically relevant ones. Therefore, it is important to consider the recognition pattern for antivenoms against individual venom components or fractions. To further investigate these immunorecognition patterns, all three antivenoms were subjected to yet another ELISA assay against the different venom fractions (Figure 6). From these results it was observed that the three antivenoms generally have a similar recognition pattern. However, the SAVP antivenom does appear to have higher antibody titers than the other antivenoms against the medically most relevant fractions containing the α -neurotoxins (fractions 3, 4, 5, and 8) (see Figure 6).

4.0 Concluding remarks and outlook

In the present study, the venom of *N. melanoleuca* was, for the first time, subjected to a thorough toxicovenomics analysis. This revealed that the venom was dominated by three-finger toxins (57.1 w% of the venom) and phospholipase A₂s (12.9 w% of the venom), of which particularly the three-finger toxins were determined to be the most toxic fractions of the venom evaluated by their Toxicity Score. Additionally, other protein families (CRISPs, nerve growth factor, Kunitz-type serine protease inhibitor, ohanin/vespryn, SVMPs, DNA/RNA non-specific endonuclease, endonuclease/phosphodiesterase, flavin monoamine oxidase, glutathione peroxidase, Type-B carboxylesterase/lipase, and selectins) were determined to be present in the venom. Immunoprofiling of three antivenoms by ELISA with the different fractions of *N. melanoleuca* venom revealed a similar pattern of immunorecognition, although the South African antivenom exhibited slightly higher signals against the

toxicologically relevant neurotoxins. Our toxicovenomic observations indicate that an effective antivenom against the venom of *N. melanoleuca* should contain neutralizing antibodies against venom components having homology to the α -neurotoxins Alpha-neurotoxin NTX-1 from *N. sputratix* (Q9YGJ6), Short neurotoxin 1 from *N. melanoleuca* (P01424), Long neurotoxin 2 from *N. melanoleuca* (P01388), and Long neurotoxin OH-55 *O. hannah* (Q53B58). Hopefully, these studies may help lay the foundation for developing more efficacious antivenoms, based on traditional or novel biotechnological approaches.

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References

- [1] P.J. Mirtschin, N. Dunstan, B. Hough, E. Hamilton, S. Klein, J. Lucas, et al.,
Venom yields from Australian and some other species of snakes,
Ecotoxicology. 15 (2006) 531–538.
- [2] R. Shine, W.R. Branch, J.K. Webb, P.S. Harlow, T. Shine, J.S. Keogh, Ecology
of cobras from southern Africa, J. Zool. 272 (2007) 183–193.
- [3] L. Luiselli, F.M. Angelici, Ecological relationships in two Afrotropical cobra
species (*Naja melanoleuca* and *Naja nigricollis*), Can. J. Zool. Can. Zool. 78
(2000) 191–198.
- [4] M. O'Shea, Venomous Snakes of the World, Princetown University Press,
2005.
- [5] World Health Organization, Guidelines for the Prevention and Clinical
Management of Snakebite in Africa, Brazzaville, World Health Organization
(2010). Available in <http://www.afro.who.int/en/clusters-a-programmes/hss/essential-medicines/edm-publications/2731-guidelines-for-the-prevention-and-clinical-management-of-snakebite-in-africa.html>
- [6] A.H. Laustsen, M. Engmark, C. Milbo, J. Johannesen, B. Lomonte, J.M.
Gutiérrez, et al., From fangs to pharmacology: The future of antivenoms, Curr.
Pharm. Des. 22 (2016).
- [7] R.A. Shipolini, G.S. Bailey, B.E. Banks, The separation of neurotoxin from the
venom of *Naja melanoleuca* and the primary sequence determination., Eur. J.
Biochem. 42 (1974) 203–11.
- [8] D.P. Botes, Snake venom toxins. The amino acid sequences of toxins b and d
from *Naja melanoleuca* venom., J. Biol. Chem. 247 (1972) 2866–71.
- [9] F.H. Carlsson, Snake venom toxins. The primary structure of protein S4C11. A

- neurotoxin homologue from the venom of forest cobra (*Naja melanoleuca*),
 Biochim. Biophys. Acta. 400 (1975) 310–21.
- [10] R.A. Shipolini, M. Kissonerghis, B.E. Banks, The primary structure of a major polypeptide component from the venom of *Naja melanoleuca*, Eur. J. Biochem. 56 (1975) 449–54.
- [11] F.H.H. Carlsson, F.J. Joubert, Snake venom toxins The isolation and purification of three cytotoxin homologues from the venom of the forest cobra (*Naja melanoleuca*) and the complete amino acid sequence of toxin VIII, Biochim. Biophys. Acta - Protein Struct. 336 (1974) 453–469.
- [12] F.J. Joubert, *Naja melanoleuca* (forest cobra) venom. The amino acid sequence of phospholipase A, fraction DE-III., Biochim. Biophys. Acta. 379 (1975) 329–44.
- [13] F.J. Joubert, The amino acid sequence of phospholipase A, fractions DE-I and DE-II., Biochim. Biophys. Acta. 379 (1975) 345–59.
- [14] F.J. Joubert, N. Taljaard, Snake venoms. The amino acid sequences of two Melanoleuca-type toxins., Hoppe-Seyler's Zeitschrift Für Physiol. Chemie. 361 (1980) 425–36.
- [15] A.H. Laustsen, B. Lohse, B. Lomonte, M. Engmark, J.M. Gutiérrez, Selecting key toxins for focused development of elapid snake antivenoms and inhibitors guided by a Toxicity Score., Toxicon. 104 (2015) 43–5.
- [16] A.H. Laustsen, J.M. Gutiérrez, B. Lohse, A.R. Rasmussen, J. Fernández, B. Lomonte, Snake venomomics of monocled cobra (*Naja kaouthia*) and investigation of human IgG response against venom toxins, Toxicon. 99 (2015) 23–25.
- [17] J. Calvete, Proteomic tools against the neglected pathology of snake bite

- envenoming, *Expert Rev Proteomics*. (2011) 739–758.
- [18] B. Lomonte, W.C. Tsai, J.M. Ureña-Díaz, L. Sanz, D. Mora-Obando, E.E. Sánchez, et al., Venomics of new world pit vipers: Genus-wide comparisons of venom proteomes across *Agkistrodon*, *J. Proteomics*. 96 (2014) 103–116.
- [19] M. Holzer, S. Mackessy, An aqueous endpoint assay of snake venom phospholipase A₂, *Toxicon*. (1996) 1149–55.
- [20] W. Wang, C. Shih, T. Huang, A novel P-I class metalloproteinase with broad substrate-cleaving activity, agkislysin, from *Agkistrodon acutus* venom, *Biochem Biophys Res Commun*. (2004) 224–230.
- [21] D. Finney, *Statistical Methods in Biological Assay*, London: Charles Griffin and Company Limited, 1971.
- [22] L.P. Lauridsen, A.H. Laustsen, B. Lomonte, J.M. Gutiérrez, Toxicovenomics and antivenom profiling of the Eastern green mamba snake (*Dendroaspis angusticeps*), *J. Proteomics*. 136 (2016) 248–261.
- [23] A.H. Laustsen, B. Lomonte, B. Lohse, J. Fernández, J.M. Gutiérrez, Unveiling the nature of black mamba (*Dendroaspis polylepis*) venom through venomics and antivenom immunoprofiling: Identification of key toxin targets for antivenom development., *J. Proteomics*. 119 (2015) 126–142.
- [24] V. Tsetlin, Snake venom alpha-neurotoxins and other “three-finger” proteins., *Eur. J. Biochem*. 264 (1999) 281–6.
- [25] R.M. Kini, R. Doley, Structure, function and evolution of three-finger toxins: Mini proteins with multiple targets, *Toxicon*. 56 (2010) 855–867.
- [26] T. Endo, N. Tamiya, Current view on the structure-function relationship of postsynaptic neurotoxins from snake venoms., *Pharmacol. Ther*. 34 (1987) 403–51.

- [27] K. Sunagar, T.N.W. Jackson, E.A.B. Undheim, S.A. Ali, A. Antunes, B.G. Fry, Three-fingered RAVeRs: Rapid Accumulation of Variations in Exposed Residues of snake venom toxins., *Toxins (Basel)*. 5 (2013) 2172–208.
- [28] C.-I.A. Wang, T. Reeks, I. Vetter, I. Vergara, O. Kovtun, R.J. Lewis, et al., Isolation and structural and pharmacological characterization of α -elapitoxin-Dpp2d, an amidated three finger toxin from black mamba venom., *Biochemistry*. 53 (2014) 3758–66.
- [29] C.M. Barber, G.K. Isbister, W.C. Hodgson, Alpha neurotoxins. *Toxicon*. 66 (2013) 47-58.
- [30] S. Nirthanan, M.C. Gwee, Three-finger alpha-neurotoxins and the nicotinic acetylcholine receptor, forty years on. *J. Pharmacol. Sci.* 94 (2004) 1-17.
- [31] M. Rivel, D. Solano, M. Herrera, M. Vargas, M. Villalta, Á. Segura, et al., Pathogenesis of dermonecrosis induced by venom of the spitting cobra, *Naja nigricollis*: An experimental study in mice., *Toxicon*. 119 (2016) 171–179.
- [32] P. V. Dubovskii, Y.N. Utkin, Cobra cytotoxins: Structural organization and antibacterial activity, *Acta Naturae*. 6 (2014) 11–18.
- [33] F.F. Davidson, E.A. Dennis, Evolutionary relationships and implications for the regulation of phospholipase A₂ from snake venom to human secreted forms, *J. Mol. Evol.* 31 (1990) 228–238.
- [34] R.M. Kini, Excitement ahead: Structure, function and mechanism of snake venom phospholipase A₂ enzymes, *Toxicon*. 42 (2003) 827–840.
- [35] K.Y. Tan, C.H. Tan, S.Y. Fung, N.H. Tan, Venomics, lethality and neutralization of *Naja kaouthia* (monocled cobra) venoms from three different geographical regions of Southeast Asia, *J. Proteomics*. 120 (2015) 105–125.
- [36] C. Montecucco, J.M. Gutiérrez, B. Lomonte, Cellular pathology induced by

- snake venom phospholipase A₂ myotoxins and neurotoxins: common aspects of their mechanisms of action., Cell. Mol. Life Sci. 65 (2008) 2897–912.
- [37] R. Shashidharamurthy, K. Kemparaju, A neurotoxic phospholipase A₂ variant: Isolation and characterization from eastern regional Indian cobra (*Naja naja*) venom, Toxicon. 47 (2006) 727–733.
- [38] F. Joubert, Taljaard N, Complete primary structure of toxin CM-1C from *Hemachatus haemachatus* (Ringhals) venom, South African J. Chem. 32 (1979) 73–77.

Figure legends

Figure 1: *Naja melanoleuca* in a raised position displaying its characteristic cobra hood.

Figure 2: Separation of *N. melanoleuca* venom proteins using RP-HPLC (A), followed by SDS-PAGE (B). Two mg of venom were fractionated on a C₁₈ column and eluted with an acetonitrile gradient (dashed line), followed by further separation of protein fractions by SDS-PAGE under reducing conditions. Molecular weight markers (M) are indicated in kDa. Coomassie-stained bands were excised, in-gel digested with trypsin, and subjected to MALDI-TOF/TOF analysis for assignment to protein families, as shown in [Table 1](#).

Figure 3: Composition of the *N. melanoleuca* venom proteome according to protein families, expressed as percentages of total protein content based on w%. **3FTx**: three-finger toxins; **PLA₂**: phospholipase A₂s; **CRISP**: cysteine-rich secretory proteins; **NGF**: Nerve growth factors; **KUN**: Kunitz-type serine protease inhibitors; **OHA**: Ohanins/vespryns; **MP**: Snake venom metalloproteinases; **DRE**: DNA/RNA non-specific endonucleases; **PDE**: Endonucleases/Phosphodiesterases; **FMO**: Flavin monoamine oxidases; **GPF**: glutathione peroxidases; **CELF**: Type-B carboxylesterases/lipases; **SUSHI**: Selectins; **UNK**: Unknown.

Figure 4: (A) Comparison of the phospholipase A₂ activity of 20 µg of the venoms of *N. melanoleuca*, *N. mossambica*, *N. nigricollis*, and *Bothrops asper* on 4-nitro-3-octanoyloxybenzoic acid synthetic substrate. (B) Comparison of the proteolytic

activity of 20 µg of venoms of *N. melanoleuca*, *N. mossambica*, *N. nigricollis*, and *Bothrops asper* on azocasein substrate. Each bar represents mean \pm SD of triplicates.

Figure 5: ELISA titrations of antivenoms against immobilized crude venoms of *N. melanoleuca*. **SAVP:** SAIMR Polyvalent Snake Antivenom from South African Vaccine Producers. **African:** Snake Venom Antiserum (African) from VINS Bioproducts Ltd. **Central Africa:** Snake Venom Antiserum (Central Africa) from VINS Bioproducts Ltd. **Control:** Normal horse serum. Each point represents mean \pm SD of triplicate wells. Antivenom titrations are represented as volumetric dilutions in (A), or as protein concentrations in (B).

Figure 6: ELISA-based immunoprofiling of antivenoms against HPLC fractions of *N. melanoleuca* venom. For identification of venom fractions see [Table 2](#). **SAVP:** SAIMR Polyvalent Snake Antivenom from South African Vaccine Producers. **African:** Snake Venom Antiserum (African) from VINS Bioproducts Ltd. **Central Africa:** Snake Venom Antiserum (Central Africa) from VINS Bioproducts Ltd. **Control:** Normal horse serum. Each bar represents mean \pm SD of triplicate wells.

Table 1: Assignment of the RP-HPLC isolated fractions of *Naja melanoleuca* venom to protein families by MALDI-TOF-TOF of selected peptide ions from in-gel trypsin-digested protein bands.

Peak	%	Mass (kDa) [▼]	Peptide ion	MS/MS-derived sequence [*]	Conf (%)	Sc	Protein family ^{**}	Related protein; access code
3a	0.7	19	m/z	z	99	8	3FTx	Alpha-neurotoxin NTX-1, <i>Naja sputatrix</i> ; Q9YGI6
			1466.7	1				
3b	6.1	14	1338.6	1	99	10	3FTx	Short neurotoxin 1, <i>Naja melanoleuca</i> ; P01424
			1229.5	1	99	7		
			1039.4	1	99	8		
			1357.6	1	99	10		
			1772.7	1	99	18		
			1497.7	1	98.6	8		
3c	0.9	11	1427.5	1	95.6	6	UNK	Unknown
				1				
4a	0.2	14	-	-	-	-	3FTx	Alpha-neurotoxin NTX-1, <i>Naja sputatrix</i> ; Q9YGI6
			1338.6	1	99	9		
4b	0.4	13	1466.7	1	96.3	6	3FTx	Alpha-neurotoxin NTX-1, <i>Naja sputatrix</i> ; Q9YGI6
			1772.7	1	99	16		
5a	0.3	15	1338.6	1	99	9	3FTx	Short neurotoxin 1, <i>Naja melanoleuca</i> ; P01424
			1772.7	1	99	9		
5b	0.8	13	1338.6	1	99	9	3FTx	Short neurotoxin 1, <i>Naja melanoleuca</i> ; P01424
			1772.7	1	99	9		
6	0.4	-	1772.7	1	99	9	UNK	Unknown
			-	-	-	-		
7a	0.8	12	2365.1	1	97.3	10	3FTx	Short-chain 3FTx 7, <i>Bungarus flaviceps</i> ; D5J9P4
			1316.5	1	99	6		
7b	0.6	12	1628.6	1	94.4	6	3FTx	Long neurotoxin 2, <i>Naja melanoleuca</i> ; P01388
			1628.6	1	99	6		
8a	3.2	12	1698.8	1	99	9	3FTx	Weak toxin S4C11, <i>Naja melanoleuca</i> ; P01400
			1317.6	1	99	12		
			1040.5	1	99	12		
			1196.6	1	99	11		
			1137.5	1	99	12		
			1137.5	1	99	12		
8a	3.2	12	1342.5	1	99	7	3FTx	Long neurotoxin OH-55, <i>O. humnudi</i> ; Q53B58
			1342.5	1	99	7		

8b	4.5	10	2472.0 1628.6	1 1	CFITPDVTSQICADGHVCYTK CCSTDNCNPFPTTR	99 99	19 19	3FTx	Long neurotoxin 2, <i>Naja melanoleuca</i> ; P01388
			1342.5	1	TWCDAWCTSR	99	19	3FTx	Long neurotoxin OH-55, <i>O. hannah</i> ; Q53B58
8c	5.5	10	2472.2 1628.7	1 1	CFITPDVTSQICADGHVCYTK CCSTDNCNPFPTTR	99 99	20 19	3FTx	Long neurotoxin 2, <i>Naja melanoleuca</i> ; P01388
			1342.6	1	TWCDAWCTSR	99	15	3FTx	Long neurotoxin OH-55, <i>O. hannah</i> ; Q53B58
9a	1.1	13	1298.6	1	TWCDAWCGSR	97	11	3FTx	Long neurotoxin OH-55, <i>O. hannah</i> ; Q53B58
			2366.1	1	SIFGVTT(E TM)DCPDGQNLCKFR	99	11	3FTx	Muscarinic toxin-like prot. 2, <i>N. kaouthia</i> ; P82463
9b	0.9	10	1342.6	1	TWCDAWCTSR	99	14	3FTx	Long neurotoxin OH-55, <i>O. hannah</i> ; Q53B58
			2197.0	1	TPETTEICPDSWYFCYK	99	25	3FTx	Muscarinic toxin-like prot. <i>B. multicinctus</i> ; Q9W727
10a	0.4	12	1298.6 1241.5	1 1	TWCDAWCGSR WCDAWCTSR	99 99	13 10	3FTx	Long neurotoxin OH-55, <i>O. hannah</i> ; Q53B58
10b	0.3	10	1342.6 1628.7	1 1	TWCDAWCTSR CCSTDNCNPFPTTR	99 99	11 16	3FTx	Long neurotoxin OH-55, <i>O. hannah</i> ; Q53B58
			1508.7	1	ITCSAEETFCYK	99	15	3FTx	Bucardin, <i>Bungarus candidus</i> ; P81782
10c	0.2	10	1342.6 1628.7	1 1	TWCDAWCTSR CCSTDNCNPFPTTR	99 99	11 9	3FTx	Long neurotoxin OH-55 <i>O. hannah</i> ; Q53B58
			1361.6	1	YVCCNTDRCN	98.1	7	3FTx	Cytotoxin 1, <i>Naja melanoleuca</i> ; P01448
			1508.7	1	ITCSAEETFCYK	99	14	3FTx	Bucardin, <i>Bungarus candidus</i> ; P81782
11	0.6	10	2218.2 1436.7	1 1	NLCYQMYMVSKSTIPVKR NLCYQMYMVSK	98.2 96.8	6 6	3FTx	Cytotoxin 1, <i>Naja melanoleuca</i> ; P01448
12a	trace	14	1415.7 1363.7 2261.0 2768.5	1 1 1 1	CKNPNEPSGCR ALTMENQASWR GHDSHWNSYCTETDTEFK TTATDIKGNITVIMENVNLDNKVYK	99 99 99 97.5	12 16 18 6	NGF	Nerve growth factor, <i>Naja naja</i> ; P01140
	trace		1813.9 1864.8	1 1	APYIDKNYNIDFNAR SWWHFADYGCCGR	99 99	16 16	PLA ₂	PLA ₂ DE-III, <i>Naja melanoleuca</i> ; P00601

12b	3.8	10	1205.6 1424.7	1 1	FKTIDECHR FCELLPAETGLCK	99 99	8 20	KUN	Kunitz-type SP inhibitor 2, <i>Naja nivea</i> ; P00986
	trace		1436.7	1	NLCYQMYMYSK	99	9	3FTx	Cytotoxin 1, <i>Naja melanoleuca</i> ; P01448
13a	2.8	13	1818.9 1241.6 1126.6	1 1 1	ATYNDKNNIDFNAR NMHICTVPINR NNYINIDFNAR	99 99 99	19 12 12	PLA ₂	PLA ₂ 1, <i>Naja melanoleuca</i> ; P00599
			1783.7	1	CCQIHIDNCYGEAEK	99	10	PLA ₂	PLA ₂ DE-III, <i>Naja melanoleuca</i> ; P00601
13b	2.0	10	1711.7 1864.8	1 1	TYTYECSQGLTCK SWWHFADYGCYCGR	99 99	8 18	PLA ₂	PLA ₂ 4, <i>Naja sagittifera</i> ; Q6T179
			1436.6 1361.5	1 1	NLCYQMYMYSK YVCCNTDRCN	99 99	15 8	3FTx	Cytotoxin 1, <i>Naja melanoleuca</i> ; P01448
14a	2.7	13	3350.4 1232.6 2953.3	1 1 1	TYTYESCOGTLTSCGAN(N ^{4b})KCAASVCDDR NMIOCTVPINR GGSGTPVDDLDRCQIHIDNCYGEAEK	99 99 96.8	8 12 7	PLA ₂	PLA ₂ DE-II, <i>Naja melanoleuca</i> ; P00600
			1813.9 1864.8	1 1	APYIDKNNIDFNAR SWWHFADYGCYCGR	99 99	24 19	PLA ₂	PLA ₂ DE-III, <i>Naja melanoleuca</i> ; P00601
14b	8.1	10	1361.5 1436.6	1 1	YVCCNTDRCN NLCYQMYMYSK	99 99	11 17	3FTx	Cytotoxin 1, <i>Naja melanoleuca</i> ; P01448
	trace		1813.9	1	APYIDKNNIDFNAR	99	8	PLA ₂	PLA ₂ DE-III, <i>Naja melanoleuca</i> ; P00601
15a	7.1	14	1123.6 2102.0 1813.9 2953.3	1 1 1 1	ISGCWPYIK APYIDKNNIDFNARCQ APYIDKNNIDFNAR GGSGTPVDDLDRCQIHIDNCYGEAEK	99 99 99 98.7	9 9 25 7	PLA ₂	PLA ₂ DE-III, <i>Naja melanoleuca</i> ; P00601
			1232.6 1864.8	1 1	NMIOCTVPINR SWWHFADYGCYCGR	99 99	12 20	PLA ₂	PLA ₂ muscarinic inhibitor, <i>Naja sputatrix</i> ; Q92084
15b	5.6	10	1988.9 2832.4 1361.5 1436.6	1 1 1 1	SSLVKYVCCNTDRCN TCPAGKNLCYQMYMYSKSTIPVKR YVCCNTDRCN NLCYQMYMYSK	99 99 99 99	8 7 11 18	3FTx	Cytotoxin 1, <i>Naja melanoleuca</i> ; P01448

16a	0.4	14	1783.7	1	CCQIHDCYGEAEK	99	12	PLA ₂	PLA ₂ DE-III, <i>Naja melanoleuca</i> ; P00601
			1123.6	1	ISGCWPYIK	99	11		
			1813.9	1	APYIDKNYNIDFNAR	99	22		
16b	1.3	10	1232.6	1	NMIQCTVPNR	99	11	PLA ₂	PLA ₂ muscarinic inhibitor, <i>Naja sputatrix</i> ; Q92084
			1050.4	1	DYGCYCGR	99	11		
			1864.8	1	SWWHFADYGCYCGR	99	20		
17	3.4	11	1361.5	1	YVCCNIDRCN	99	11	3FTx	Cytotoxin 1, <i>Naja melanoleuca</i> ; P01448
			1436.6	1	NLCYQMYMWSK	99	17		
			1646.9	1	IKCHNTLLPFIYK	99	19	3FTx	Cytotoxin homolog 2, <i>Naja melanoleuca</i> ; P01474
			1353.6	1	TCPEGQNLCFK	99	18		
18	4.2	10	1405.7	1	CHNTLLPFIYK	99	17		
			2125.1	1	TCPEGQNLCFKGTLKFPK	99	7	3FTx	Cytotoxin homolog 2, <i>Naja melanoleuca</i> ; P01474
			1353.6	1	TCPEGQNLCFK	99	16		
			1646.9	1	IKCHNTLLPFIYK	99	18		
19a	0.1	24	1405.7	1	CHNTLLPFIYK	99	17		
			2768.4	1	TTATDIKGNTVTVMENVNLDNKVYK	99	6	NGF	Nerve growth factor, <i>Naja naja</i> ; P01140
			1415.6	1	CKNPPEPSGCR	99	10		
			2261.0	1	GIDSSHWSYCTETDTFIK	99	21		
19b	0.3	14	1363.7	1	ALTMENQASWR	99	16		
			2768.4	1	TTATDIKGNTVTVMENVNLDNKVYK	99	11	NGF	Nerve growth factor, <i>Naja naja</i> ; P01140
			1415.6	1	CKNPPEPSGCR	99	12		
			2261.0	1	GIDSSHWSYCTETDTFIK	99	27		
			1363.7	1	ALTMENQASWR	99	17		
			1824.0	1	FIRIDTACVCVITKK	97.7	8		
19c	0.4	11							
			1864.7	1	SWWHFADYGCYCGR	99	12	PLA ₂	PLA ₂ B, <i>Naja sputatrix</i> ; Q92085
			3013.3	1	FTCFPTPSDTSCTCPDGQNICYEKR	99	9	3FTx	Weak toxin CM-2, <i>Naja huaje</i> ; P01415
			2857.1	1	FTCFPTPSDTSCTCPDGQNICYEK	66.8	8		
20a	0.5	14	2270.1	1	ADVTFDSNTAFESLVVSPDKK	99	20	OHA	Thaicobrin, <i>Naja kaouthia</i> ; P82885
			2595.3	1	SGKHFEVVKYGTQREWAVGLAGK	99	10		
			3286.6	1	TVENVGVSOVAPDNPERFDGSPCVLGSPGFR	99	13		
			1535.8	1	YGTQREWAVGLAGK	99	17		
20b	1.0	11	1810.9	1	TVENVGVSOVAPDNPER	99	24		
			3013.3	1	FTCFPTPSDTSCTCPDGQNICYEKR	99	9	3FTx	Weak toxin CM-2, <i>Naja huaje</i> ; P01415

				2857.1	1	FTCFITPSDITSETCPDGQNICYEK	99	11		
21a	0.3	31	1441.7	1	WSNLP(T th)LCQGR	99	12	SUSHI	C' decay-accelerating factor, <i>O. hannah</i> ; ETE59511	
21b	4.5	11	3013.3	1	FTCFITPSDITSETCPDGQNICYEK	99	11	3FTx	Weak toxin CM-2, <i>Naja huaje</i> ; P01415	
			2857.2	1	FTCFITPSDITSETCPDGQNICYEK	99	12			
222	0.6	15	1424.8	1	KKYIWEWTDTR	99	11	CTL	CTL LP-Pse-6, <i>Pseudonaja modesta</i> ; R4G314	
			1168.6	1	YIWEWTDTR	99	10			
23	0.8	23	1349.8	1	QIVDKHNALRR	99	9	CRISP	CRISP, <i>Micropechis ikahekar</i> ; A0A024AX20	
			1849.9	1	NMLQMEWNSNAAQNAK	99	16			
			2006.0	1	NMLQMEWNSNAAQNAKR	99	17			
			1031.4	1	CAASCFCR	95.2	8			
			1826.9	1	WADRCSEFAHSPSHLR	99	7	CRISP	Latisemin, <i>Laticauda semifasciata</i> ; Q8JI38	
			1298.6	1	CSFAHSPSHLR	99	16			
24a	0.9	84	1890.8	1	LQPHAQCDSEECCEK	99	11	MP	Attrase-A, <i>Naja atra</i> ; D5LMJ3	
			1087.6	1	EHQEYLLR	99	10		SVMP-Aca-4, <i>Acanthophis wellsi</i> ; R4G2D3	
			1497.8	1	ERPQCLNKPSR	99	13			
			1388.7	1	YIEFYVVVDNK	99	13			
24b	0.4	24	1298.7	1	CSFAHSPSHLR	99	8	CRISP	Latisemin, <i>Laticauda semifasciata</i> ; Q8JI38	
			2014.0	1	VLEGIQCGESITYMISSNAR	99	10		Natrin-1, <i>Naja atra</i> ; Q7T1K6	
25	6.4	24	1451.8	1	QKEIVDLHNSLR	99	13	CRISP	Ophanin, <i>Ophiophagus hannah</i> ; Q7ZT98	
			1168.6	1	NVDENSESTR	99	12			
26a	1.5	58	1720.8	1	AAKDDCDLPELCTGR	99	14	MP	SVMP 1, <i>Micruurus fulvius</i> ; U3EPC7	
			1416.7	1	YIEFYVVVDNR	99	12			
			1544.8	1	KYIEFYVVVDNR	99	14			
			1471.7	1	TAPAFQFSSCSIR	99	17	MP	SVMP kaouthingin, <i>Naja kaouthia</i> ; P82942	
26b	0.5	44	1720.8	1	AAKDDCDLPELCTGR	99	15	MP	SVMP 1, <i>Micruurus fulvius</i> ; U3EPC7	
			1544.9	1	KYIEFYVVVDNR	94.2	6			
			1471.7	1	TAPAFQFSSCSIR	99	17	MP	Attrase-B, <i>Naja atra</i> ; D6PXE8	
26c	0.3	29	2110.1	1	GCOQTFAVVGAVPGDTYIAR	99	24	DRE	Endonuc domain-containing 1 prot <i>O. hannah</i> ; V8N4Y2	
			1634.8	1	GHLNPNGHQPDYSAK	99	16			

			2243.0	1	LNGGAWNNYEQTTMQQMT	99	13		
			2024.0	1	EVVDSFQDHCPQFLLR	99	19	DRE	Endonuclease domain-containing 1 prot <i>M. fulvius</i> ; U3FCT9
27a	1.2	73	1087.6	1	EHQEXLLR	99	12	MP	Atrase-A, <i>Naja atra</i> ; D5LMJ3
			1646.8	1	VYEM ⁶⁹ YNALNTMYR	98.1	7	MP	Mocarhagin, <i>Naja mossambica</i> ; Q10749
27b	0.7	54	1720.8	1	AAKDDCDLPELCTGR	99	8	MP	SVMP 1, <i>Micrurus fulvius</i> ; U3EPC7
			1416.7	1	YIEFYVVVDNR	99	13		
			1544.8	1	KYIEFYVVVDNR	99	13		
			1087.6	1	EHQEXLLR	99	6	MP	Atrase-A, <i>Naja atra</i> ; D5LMJ3
27c	0.2	49	1416.8	1	YIEFYVVVDNR	99	11	MP	MTP4, <i>Micrurus fulvius</i> ; U3FWL3
			1544.8	1	KYIEFYVVVDNR	99	12		
			1087.6	1	EHQEXLLR	99	9	MP	Atrase-A, <i>Naja atra</i> ; D5LMJ3
27d	0.3	41	1720.8	1	AAKDDCDLPELCTGR	99	10	MP	SVMP 1, <i>Micrurus fulvius</i> ; U3EPC7
			1416.8	1	YIEFYVVVDNR	99	12		
			1544.9	1	KYIEFYVVVDNR	98.3	9		
			1087.6	1	EHQEXLLR	99	9	MP	Atrase-A, <i>Naja atra</i> ; D5LMJ3
28a	1.1	118	1518.8	1	LWNFYHSTLLPK	99	9	PDE	Phosphodiesterase 1, <i>Micrurus fulvius</i> ; U3FAB3
			2106.0	1	RPDESTLYIEPDTGHHK	99	17		
28b	0.2	73	1087.6	1	EHQEXLLR	99	6	MP	Atrase-A, <i>Naja atra</i> ; D5LMJ3
29a	0.2	71	1224.7	1	ALAEMVHLNAR	98.9	7	FMO	L-aminooxidase, <i>Naja atra</i> ; A8QL58
29b	0.2	30	1329.7	1	IHDIKWNFEK	99	13	MP	Glutathione peroxidase, <i>O. hannah</i> ; V8P395
			1492.8	1	LVILGFPENQFGK	99	8		
			1385.8	1	FLVNPQGGKPYMR	99	12		
29c	0.7	25	1492.8	1	LVILGFPENQFGK	99	12	MP	Glutathione peroxidase, <i>O. hannah</i> ; V8P395
			1385.8	1	FLVNPQGGKPYMR	99	11		
30a	0.2	250	1344.7	1	KRWIADGHHGK	99	9	FMO	L-aminooxidase, <i>Naja atra</i> ; A8QL58
			1224.7	1	ALAEMVHLNAR	99	12		

30b	0.6	74	1539.8	1	NNKVYAYLFDHR	99	8	CELF	ACN-Den-1, <i>Denisonia devisi</i> ; R4FJM1
			2390.2	1	AVTTFGESAGAASVGMHLLSTQSR	99	10		
			2137.1	1	ALLQSGAPNAPWATVTPAESR	99	18	CELF	Acetylcholinesterase 1, <i>O. aestivus</i> ; A0A098LYD4
30c	1.0	62	1017.6	1	VATQTGWVR	99	8	CELF	Acetylcholinesterase 2, <i>Python regius</i> ; A0A098LWS5
			1224.7	1	ALAEMVHLNAR	99	15	FMO	L-amino-acid oxidase, <i>Naja atra</i> ; A8QL58
			2546.2	1	SPLEECF(R ^{en})EADYEELIAR	99	13	FMO	L-amino-acid oxidase, <i>B. multicitinctus</i> ; A8QL51
31a	0.6	76	2137.1	1	ALLQSGAPNAPWATVTPAESR	82.7	5	CELF	Acetylcholinesterase 1, <i>Echis coloratus</i> ; A0A0A1WDT1
31b	0.5	68	1216.6	1	FWEADGHHGK	99	8	FMO	L-amino-acid oxidase, <i>Naja atra</i> ; A8QL58
			1344.7	1	KFEWADGHHGK	99	7		
			1224.7	1	ALAEMVHLNAR	99	13		
31c	0.6	62	1935.0	1	TLSTVVTADYVIVCSTSR	99	8	FMO	LAO-Hop-2, <i>H. bungaroides</i> ; R4FK16
			1344.7	1	KFEWADGHHGK	99	6		
			2517.2	1	STSMVAITMAHQMGHNLGMNDDR	99	7	MP	Mocarhagin, <i>Naja mossambica</i> ; Q10749
32a	0.9	64	1754.8	1	AAK(N ^{db})DCDFPELCTGR	99	15		
			1010.5	1	CPTDSFQR	99	9	MP	SVMP 1, <i>Micrurus fulvius</i> ; U3EPC7
			1206.7	1	(N ^{db})YDAFLGKWR	98.7	9	FABP	Fatty acid-binding protein, <i>M. fulvius</i> ; U3FZ13
32b	0.5	56	1754.8	1	AAKNDCDFPELCTGR	99	12	MP	Mocarhagin, <i>Naja mossambica</i> ; Q10749
			1010.5	1	CPTDSFQR	99	7	MP	SVMP 1, <i>Micrurus fulvius</i> ; U3EPC7
			1087.6	1	EHQEYLLR	99	8	MP	Atrase-A, <i>Naja atra</i> ; D5LMI3
33	2.8	52	1476.8	1	CPIMTNQCIALLR	99	13	MP	
			1140.6	1	DSCFTLNQR	99	11		
			1915.1	1	CPIMTNQCIALLRGPVK	98.3	7		
			1687.9	1	RTKPAY(Q ^{db})FSSCSVR	95.4	9		
			1087.6	1	EHQEYLLR	99	12	MP	Atrase-A, <i>Naja atra</i> ; D5LMI3

* Cysteine residues are carbamidomethylated. Confidence (Conf) and Score (Sc) values are calculated by the Paragon algorithm of ProteinPilot®. ▼ : reduced SDS-PAGE mass estimations, in kDa. Possible, although unconfirmed/ambiguous amino acid modifications suggested by the automated identification software are shown in parentheses, with the following abbreviations: ^{de}: deamidated; ^{na}: Na cation; ^{dh}: dehydrated; ^{ca}: carbamylated.

** Protein family abbreviations: 3FTx: Three-finger toxin; PLA₂: Phospholipase A₂; CRISP: Cysteine-rich secretory protein; NGF: Nerve growth factor; KUN: Kunitz-type serine protease inhibitor; OHA: Ohanin/vespyrin; MP: Metalloproteinase; DRE: DNA/RNA non sp-endonuclease; CTL: C-type lectin; PDE: Endonuclease/Phosphodiesterase; FMO: Flavin monoamine oxidase; GPF: Glutathione peroxidase; CELF: Type-B carboxylesterase/lipase; SUSHI: Selectins – cell adhesion (<http://pfam.xfam.org/family/PF00084>); UNK: Unknown.

Table 2: Lethality and Toxicity Score of RP-HPLC fractions of the venom of *N. melanoleuca*

Peak	w%	mol%	Protein family	LD ₅₀ (95% conf. limits)	Reported LD ₅₀ (mg/kg)	Toxicity score w% / LD ₅₀ (kg/mg) ¹	Molecular Toxicity score mol% / LD ₅₀ (kg/mg) ¹
Whole venom	100	100		0.66 (0.49-0.92)	0.289*	151	151
3	7.7	10.2	3FTx Alpha-neurotoxin NTX-1 <i>Naja sputatrix</i> ; Q9YGJ6 Short neurotoxin 1 <i>Naja melanoleuca</i> ; P01424	0.13 (0.06-0.34)		59.2	78.8
4	0.6	0.9	3FTx Alpha-neurotoxin NTX-1 <i>Naja sputatrix</i> ; Q9YGJ6 Short neurotoxin 1 <i>Naja melanoleuca</i> ; P01424	0.13 (0.05-0.33)		4.6	6.9
5	1.1	1.6	3FTx Alpha-neurotoxin NTX-1 <i>Naja sputatrix</i> ; Q9YGJ6 Short neurotoxin 1 <i>Naja melanoleuca</i> ; P01424	0.11 (0.03-0.23)		10	15.0
7	1.4	1.9	3FTx Short-chain 3FTx 7 <i>Bungarus flaviceps</i> ; D5J9P4 Long neurotoxin 2 <i>Naja melanoleuca</i> ; P01388	>0.53		<2.6	<3.6
8	13.2	17.3	3FTx Weak toxin S4C11 <i>Naja melanoleuca</i> ; P01400 Long neurotoxin OH-55 <i>O. hannah</i> ; Q53B58 Long neurotoxin 2 <i>Naja melanoleuca</i> ; P01388	0.15 (0.06-0.42)	>20 (P01400) [9]	88	115.5
9	2.0	2.7	3FTx 3FTx-Den-15 <i>Denisonia devisi</i> ; R4FID4 Muscarinic toxin-like prot. 2 <i>N. kaouthia</i> ; P82463 Long neurotoxin OH-55 <i>O. hannah</i> ; Q53B58 Muscarinic toxin-like prot <i>B. multirictus</i> ; Q9W727	>0.53		<3.8	<5.0

10	0.9	1.2	3FTx 3FTx-Den-15 <i>Denisonia devisi</i> ; R4FID4 3FTx-Ech-35 <i>Echiopsis curta</i> ; R4G7H1 Long neurotoxin OH-55 <i>O. hamadi</i> ; Q53B58 Bucandin <i>Bungarus candidus</i> ; P81782 Cytotoxin 1 <i>Naja melanoleuca</i> ; P01448	>0.53	1.36 (P01448) [11]	<1.7	<2.2
11	0.6	0.9	3FTx Cytotoxin 1 <i>Naja melanoleuca</i> ; P01448	>0.53	1.36 (P01448) [11]	<1.1	<1.7
12	3.8	6.0	Kunitz-type SP inhibitor 2 <i>Naja nivea</i> ; P00986	>0.53		<7.2	<11.4
13	4.8 (1:1 mix)	5.2 (1:1 mix)	PLA₂ PLA ₂ 1 <i>Naja melanoleuca</i> ; P00599 PLA ₂ DE-III <i>Naja melanoleuca</i> ; P00601 PLA ₂ 4 <i>Naja sagittifera</i> ; Q6T179 3FTx Cytotoxin 1 <i>Naja melanoleuca</i> ; P01448	>0.53	1.36 (P01448) [11]	<9.1	<9.9
14	10.8 (1:3 mix)	8.3 (1:3 mix)	PLA₂ PLA ₂ DE-II <i>Naja melanoleuca</i> ; P00600 PLA ₂ DE-III <i>Naja melanoleuca</i> ; P00601 3FTx Cytotoxin 1 <i>Naja melanoleuca</i> ; P01448	>0.53	1.36 (P01448) [11]	<20.4	<15.6
15	12.7 (1:1 mix)	14.0 (1:1 mix)	PLA₂ PLA ₂ DE-III <i>Naja melanoleuca</i> ; P00601 PLA ₂ muscarinic inhibitor <i>Naja sputatrix</i> ; Q92084 3FTx Cytotoxin 1 <i>Naja melanoleuca</i> ; P01448	>0.53	1.36 (P01448) [11]	<24.0	<26.5
16	1.7 (1:3 mix)	2.3 (1:3 mix)	PLA₂ PLA ₂ DE-III <i>Naja melanoleuca</i> ; P00601 PLA ₂ muscarinic inhibitor <i>Naja sputatrix</i> ; Q92084 3FTx Cytotoxin 1 <i>Naja melanoleuca</i> ; P01448	>0.53		<3.2	<4.4

17	3.4	5.1	3FTx Cytotoxin homolog 2 <i>Naja melanoleuca</i> ; P01474	>0.53	<6.4	<9.6
18	4.2	6.3	3FTx Cytotoxin homolog 2 <i>Naja melanoleuca</i> ; P01474	>0.53	<7.9	<11.8
19	0.7 (1:1 mix)	0.8 (1:1 mix)	NGF Nerve growth factor <i>Naja naja</i> ; P01140 Nerve growth factor <i>Naja naja</i> ; P01140 3FTx Weak toxin CM-1c <i>Hhaemachatus</i> ; P25676	>0.53	54 (P25676) [38]	<1.3 <1.5
20	1.5 (1:2 mix)	1.9 (1:2 mix)	OHA Thaicobrin <i>Naja kaouthia</i> ; P82885 3FTx Weak toxin CM-1c <i>Hhaemachatus</i> ; P25676	>0.53	54 (P25676) [38]	<2.8 <3.6
21	4.8	6.7	SUSHI C'-decay-accelerating factor <i>O hannah</i> ; ETE59511 C'-decay-accelerating factor <i>O hannah</i> ; V8NC63 Weak toxin CM-2 <i>Naja haje</i> ; P01415	>0.53	<9.1	<12.7
22	0.6	0.4	CTL CTL LP-Pse-6 <i>Pseudonaja modesta</i> ; R4G314	>0.53	<1.1	<0.7

[†]Toxicity Score is defined as the ratio of protein fraction abundance (%) in the venom to its estimated median lethal dose (LD₅₀), whereas Molecular Toxicity Score corresponds to the ratio of protein abundance in molar terms (%) to LD₅₀. In the case of crude venom, the % abundance was 100%.

* : <http://snakedatabase.org/pages/LD50.php#legendAndDefinitions>

Figure 1

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Figure 2
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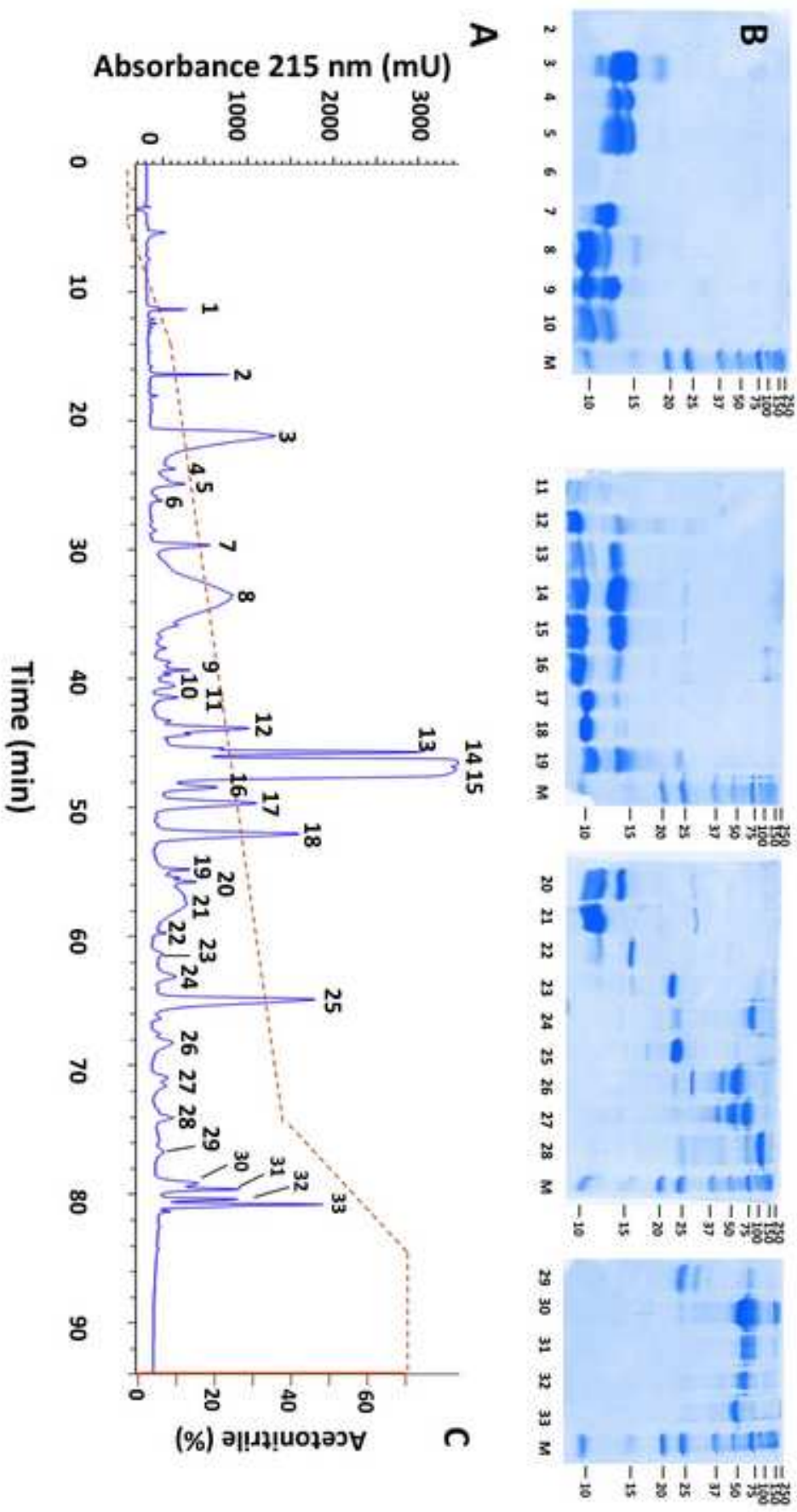


Figure 3
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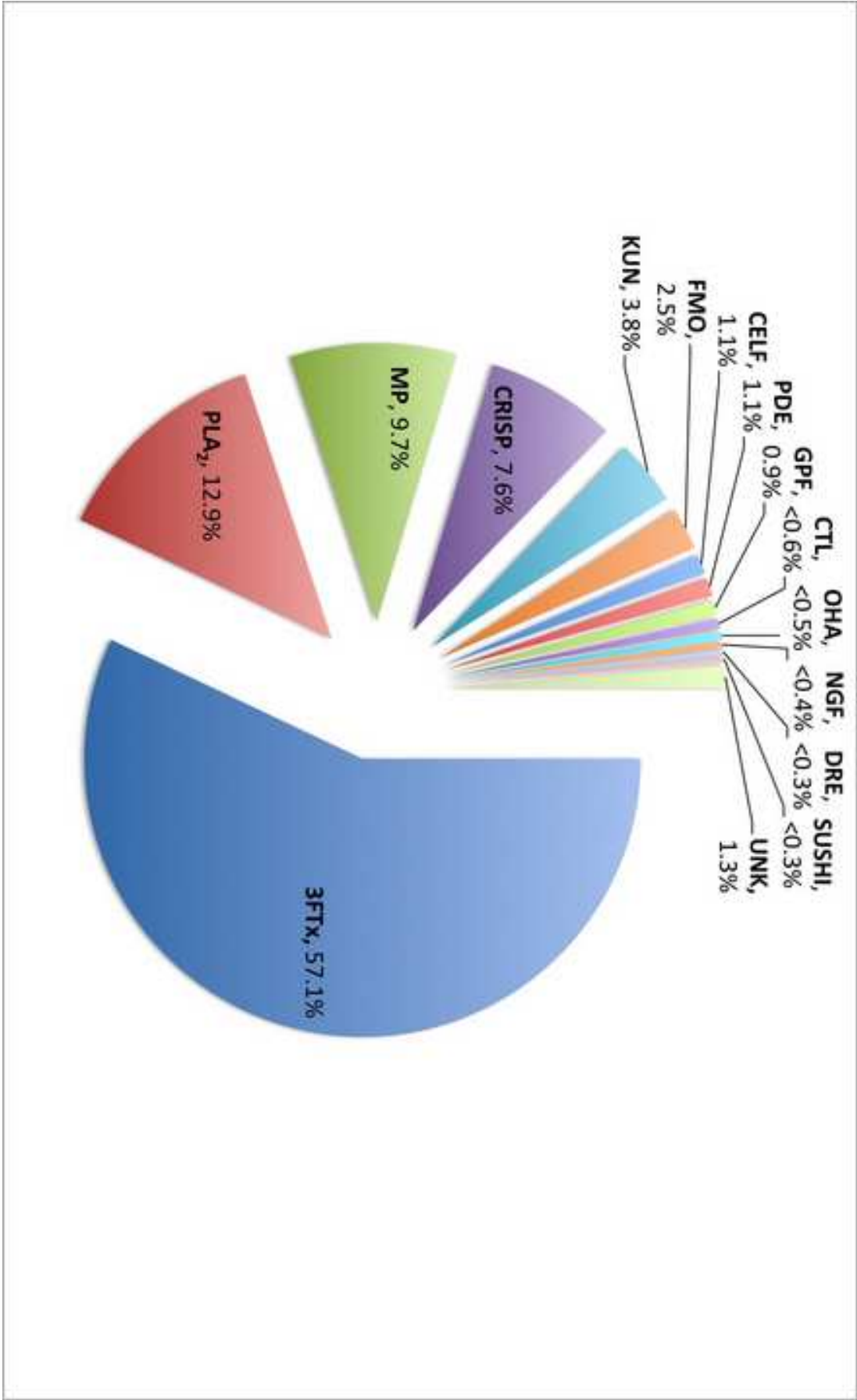


Figure 4
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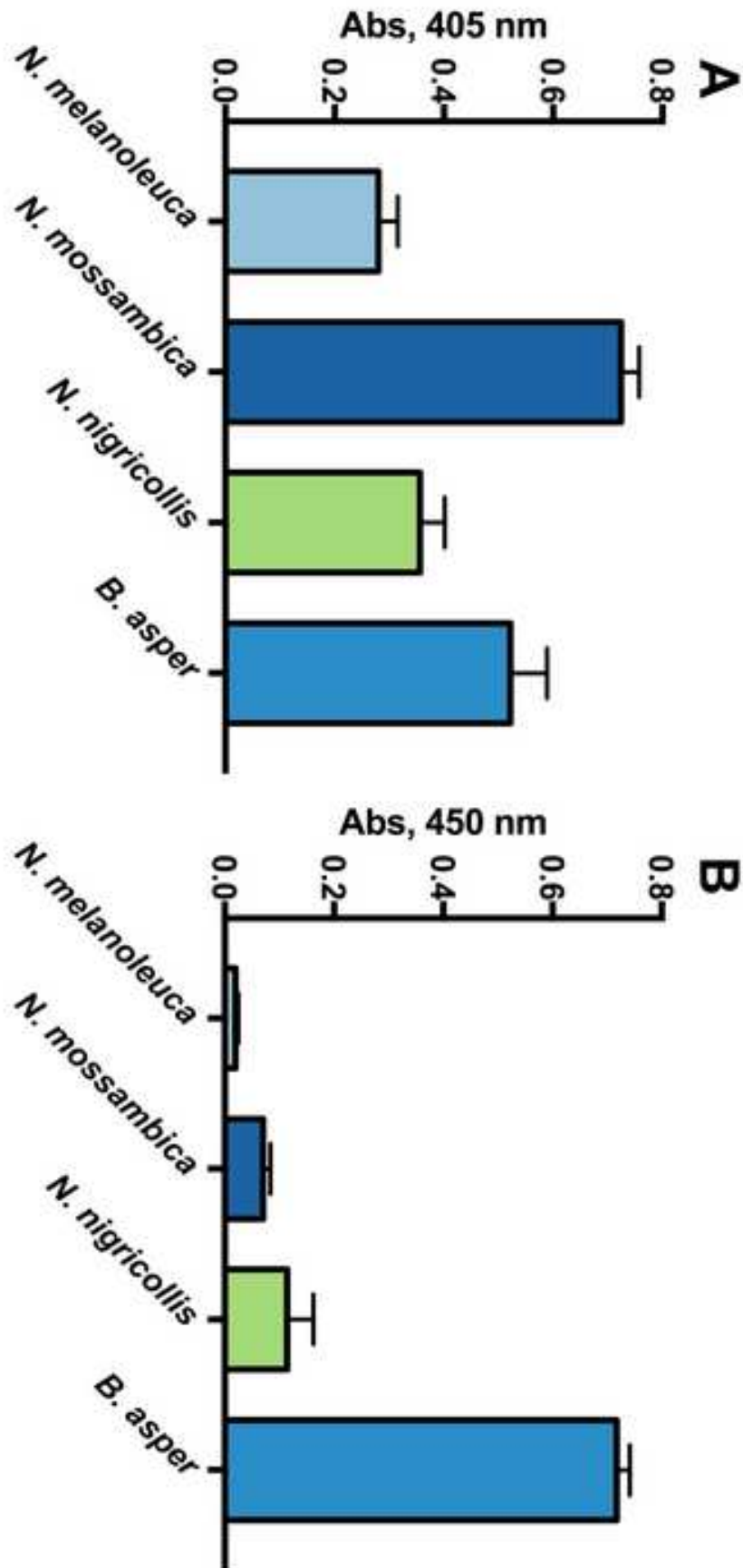


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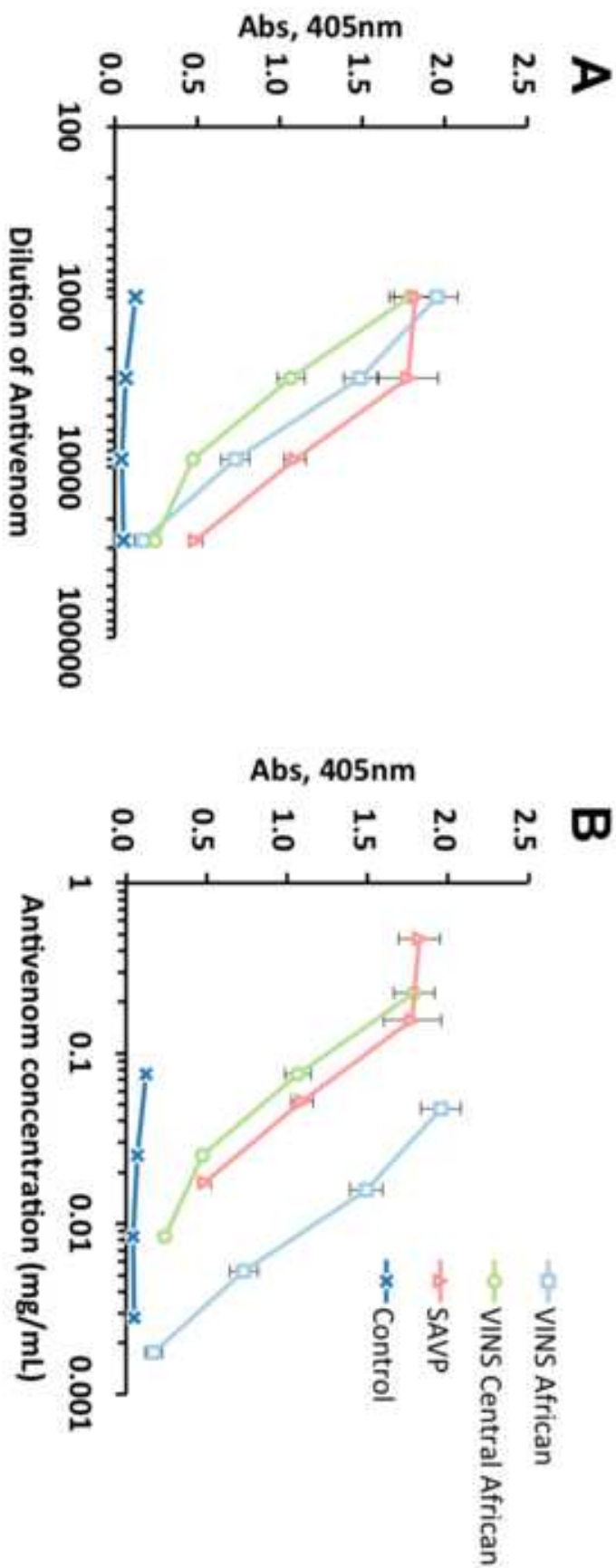
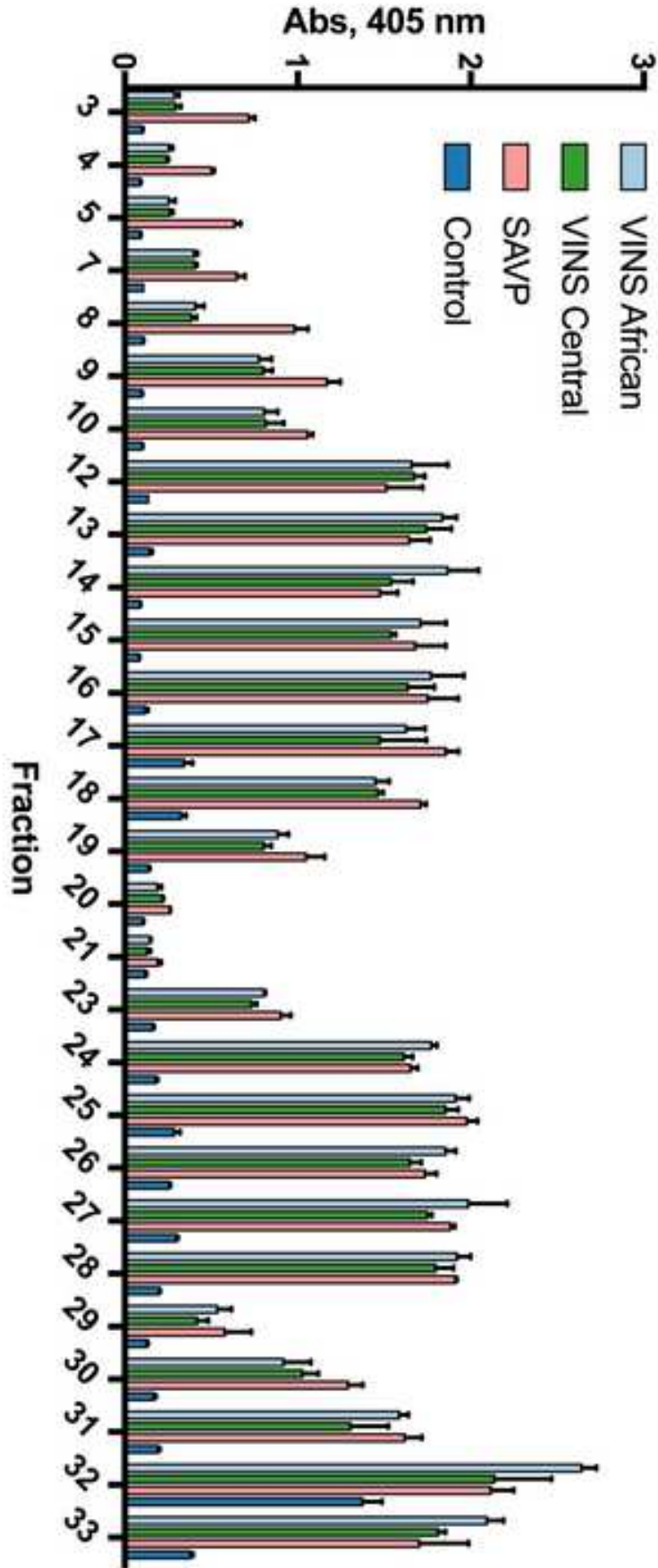


Figure 6
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